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THE BLOOD CELLS AND TUNIC OF THE ASCIDIAN *HALOCYNTHIA AURANTIUM* (PALLAS). II. HISTOCHEMISTRY OF THE BLOOD CELLS AND TUNIC[1]

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In the first paper of this series (Smith, 1969), the morphology of the tunic and blood cells of the ascidian *Halocynthia aurantium* (Pallas) was described. Of the ten blood cell types recognized in this species, four are involved in the tunic: the mature morula cell, the dispersed vesicular cell, the hyaline amoebocyte, and the granular amoebocyte. The mature morula cell and dispersed vesicular cell appear to be intimately involved with tunic growth and repair. The hyaline amoebocyte is a phagocyte. Suggestions have been made concerning the function of these blood cell types in the tunic and their interrelationships based on quantitative considerations of the distribution of these cells within and between animals.

It has been suggested that blood cells can secrete tunic (Herdman, 1899; Ries, 1937; Endean, 1955a, 1955b, 1960, and 1961), or that blood cells have an organizational, rather than secretory, function in the tunic (St. Hilaire, 1931; Das, 1936), or that the tunic is secreted primarily by the epidermis (Deck, Hay and Revel, 1966).

There have been numerous reports of the presence of cellulose in ascidian tunic (reviewed by Seeliger and Hartmeyer, 1911; Spence and Richards, 1940; Tsuchiya and Suzuki, 1963) and more recently acid mucopolysaccharides and protein have been reported (Hall and Saxl, 1960 and 1961; Bierbauer and Vagas, 1962; Godeaux, 1963).

Many of the suggestions for the origin of the tunic are based on histochemical analogy between the tunic and epidermis or tunic and blood cells. There is reason to doubt the specificity of the histochemical and fixation methods of some of the older work in light of recent reports on the nature and specificity of particular histochemical methods (Hale, 1957; Curran, 1961; Quintarelli, 1968).

This study was undertaken to ascertain the histochemical composition of the tunic, epidermis, and blood cells involved in the tunic of *H. aurantium* in order to clarify the origin of tunic and the function of the blood cells in the tunic.

Materials and Methods

The ascidians were collected and held in the laboratory as previously described (Smith, 1969).

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For routine fixation buffered formalin with cetyl pyridinium bromide in distilled water (Culling, 1963) was used. Other fixatives employed were 5% formalin in sea water, Bouin's, Baker's, Carnoy's (Culling, 1963), and N-methyl morpholine with cyanuric chloride in methanol (Goland, Nicholas and Kazimieras, 1967). Tissues were fixed for four hours in Carnoy's and for twenty-four hours in all other fixatives.

After fixation, tissues were washed in water, dehydrated in graded ethanols, cleared in benzene, embedded in Para-plast (M.P. 55.6° C), and 8 micron serial sections were cut on a Spencer microtome.

Animals less than two grams wet weight were used for histochemical preparations. The use of small whole animals facilitates the simultaneous histochemical examination of both blood cells and tunic within a single animal. All histochemical tests were performed on tissues from at least two animals and replicate slides were made from each animal.

For the detection of carbohydrates, the following procedures were employed: alcian blue with neutral red, alcian blue with periodic Schiff reaction, periodic Schiff reaction (P.A.S.), Gomori's aldehyde fuchsin (Culling, 1963), toluidine blue, methylene blue extinction test with veronal acetate buffers at approximately 0.50 pH increments from pH 1.63 to pH 9.05, Hale's dialyzed iron method (colloidal iron) (Pearse, 1961), and azure A at pH 1.5 and 4.0 (Szirmai, 1963). Some sections were methylated or methylated and saponified prior to staining with alcian blue, colloidal iron, aldehyde fuchsin, toluidine blue, and azure A (Culling, 1963). Controls for the P.A.S. reaction were filter paper and cotton gauze as well as known mucinous structures in the ascidians.

Proteins were detected histochemically by the Millon reaction, the dihydroxydinaphthyl-disulphide (DDD) method for sulfhydryls of Barnett and Seligman, and the mercury bromphenol blue method of Bonhag (Pearse, 1961). The presence of basic, histone-like proteins was determined with Bierbrich's scarlet (Spicer, 1962).

R.N.A. was stained by the methyl green-pyronin method with parallel control sections incubated in ribonuclease (Nutritional Biochemicals) (Pearse, 1961).

In order to elucidate the chemical structure of tunic, as measured by histochemical means, some tunic was treated with either chemical agents or enzymes to ascertain their effect on tunic staining properties. Prior to such treatments tunic was washed with distilled water, ethanol, acetone, and ether and then dried for 48 hours *in vacuo* over phosphorous pentoxide and sodium hydroxide.

Dried tunic was submitted to one of two chemical treatments: 8 hours hydrolysis in 6 *N* HCl under anaerobic conditions at 100° C, or 24 hours in 1 *N* HCl at 8° C followed by 36 hours in 1 *N* NaOH at 100° C. The second method parallels the procedure for the purification of lobster chitin (Hackman, 1954).

Tunic was treated with pronase, hyaluronidase, pronase followed by hyaluronidase, or a combination of papain and trypsin. The pronase (California Biochemicals) incubation mixture consisted of 0.5 mg of enzyme per mg of dried tunic in 0.15 *M* phosphate buffer at pH 8.0. The buffer was 0.005 *M* in $CaCl_2$. The digestion period was 48 hours (Barker and Young, 1966; Inoue and Yosizawa, 1966). Bovine testes hyaluronidase (California Biochemicals) medium con-

sisted of 0.2 *M* phosphate buffer at pH 6.8 with an enzyme to substrate ratio (by weight) of 1 : 10 (Dorfman, 1955). Tunic was treated with hyaluronidase for 14 days at 37° C as was tunic which had been pretreated with pronase for 48 hours. Sections of tunic also were incubated with hyaluronidase for 24 hours according to the method of Culling (1963).

Tunic was also digested with papain (Schiller, Slover and Dorfman, 1961) followed by digestion with trypsin (Schiller, Mathews, Jefferson, Ludowieg and Dorfman, 1954).

Results and Discussion

1. Histochemistry of blood cells in circulation

Conventional methods of staining for neutral and acidic polysaccharides indicate that the mature morula (Table I), immature morula cells, compartment cells, and signet ring cells do not contain these substances. The vesicles of the dispersed vesicular cells will react positively to P.A.S. only when fixed with N-methyl morpholine and cyanuric chloride (Table I). Both the mature morula cells and dispersed vesicular cells are involved in the tunic (Smith, 1969). The mature morula cells, which accumulate iron (Smith, 1969), are analogous to vanadocytes and ferrocytes (Webb, 1939; Endean, 1955a, and 1960), but unlike these cells do not stain for carbohydrate (Endean, 1955a and 1960). The dispersed vesicular cell of *Halocynthia* may be analogous to the tunic cell of *Ascidia aspersa* which takes up radioiodine and which is incorporated into the cuticle (Kennedy, 1966).

Table I

The histochemical reactions of the mature morula and dispersed vesicular cells (D.V.) in both the blood and tunic are summarized. The following convention was utilized to express intensity of staining: − negative, ± faint positive, + definitely positive, ++ strong positive, and +++ intense positive. Alpha refers to an alpha metachromasia or orthochromasia. The reactions noted are the reactions of the vacuoles of the mature morula cell and the vesicles of the despersed vesicular cell.

Reaction	Mature Morula		D.V. Cell	
	Blood	Tunic	Blood	Tunic
P.A.S.	−	−	+	+
Alcian blue	−	−	+ to −	−
Toluidine blue	−	± to − (alpha)	−	−
Colloidal iron	−	−	+	−
Azure A pH 1.5	−	−	−	−
Azure A pH 4.0	±	++ to ±	+	−
Aldehyde fuchsin	+	++ to ±	+ to −	−
Methylene blue extinction (pH)	9.05	9.05	5.00	5.35
Methyl green pyronin (RNA)	−	−	± to −	−
DDD Sulfhydryl	−	−	−	−
Bierbich's scarlet	± to −	−	+	−
Mercury bromophenol blue	+	+	+	−
Millon's	±	±	±	±

It has been suggested that in *Halocynthia* the histogenetic pathway for the differentiation of the mature morula cell type passes from the signet ring cell through the compartment cell and immature morula to the mature morula cell (Smith, 1969); this sequence parallels the hypothesis of Endean (1955a and 1960) concerning the histogenesis of analogous cell types in *Pyura stolonifera* and *Phallusia mammillata*. The consistent positive reaction of the vacuoles of the signet ring, compartment, immature morula, and mature morula cells to mercury bromphenol blue, Biebrich's scarlet, and iron stains (Smith, 1969) indicates that these cells are chemically related. The presence of accumulated iron and protein is a property which they share with the ferrocyte of *Pyura stolonifera* (Endean, 1955a).

In the blood both the hyaline amoebocytes and granular amoebocytes stain with P.A.S. (Table II). The staining is diffuse in hyaline amoebocytes, but concentrated in the granules of the granular amoebocyte. The hyaline amoebocyte is a phagocyte (Smith, 1969), but no function has been assigned to the granular amoebocyte although it, and the hyaline amoebocyte, are present in the tunic of *Halocynthia* (Smith, 1969). Both of these cells are negative to acid mucopolysaccharide stains.

Of the blood cells, the stem cell, the hyaline amoebocyte, some signet ring cells, stem cells with acidophilic vacuoles and granules, granular amoebocytes, and some dispersed vesicular cells have ribonuclease labile affinity for pyronin in the methyl green-pyronin technique for R.N.A. If R.N.A. content, as judged by pyronin affinity, can be used as a criterion of synthetic activity, then the signet ring cell probably represents the intermediate between the stem cell with acidophilic vacuoles and granules and the compartment cell.

II. Histochemistry of the mature morula and dispersed vesicular cells in the tunic

The histochemical reactions of the mature morula and dispersed vesicular cells in the tunic are summarized in Table I.

Although the mature morula cell retains its negative reaction to polysaccharide stains, it does display a variety of reactions in the tunic, as contrasted with the

TABLE II

The histochemical reactions of the hyaline amoebocyte and granular amoebocyte in the blood of H. aurantium. *The reaction of the cells are indicated as follows*: + *definitely positive*, ± *faintly positive*, − *negative. Alpha refers to an orthochromatic reaction*

Reaction	Hyaline Amoebocyte	Granular Amoebocyte
P.A.S.	+	+
Alcian blue	−	−
Toluidine blue	± (alpha)	−
Aldehyde fuchsin	−	+ to −
Methylene blue extinction (pH)	5.00	5.00
Methyl green pyronin (R.N.A.)	+ to −	+
Millon's	±	±

blood, which indicate that some chemical change is taking place in the cell prior to its degradation to the *tunic vesicular cell.* For example, the mature morula cells in the tunic show a variety of reactions to azure A at pH 4.0. This variability is also true when the cells are stained with aldehyde fuchsin. The *tunic vesicular cells,* which are degraded mature morula cells in the tunic (Smith, 1969), only rarely have an affinity for the histochemical stains used in this study. The mature morula cell in the tunic differs from the blood mature morula in its staining reactions to aldehyde fuchsin, Biebrich's scarlet, azure A at pH 4.0 (Table I), and Perl's method for iron (Smith, 1969). The *tunic vesicular cell* differs from the tunic mature morula cell in that it displays occasional reactions with colloidal iron, shows a variety of reactions to aldehyde fuchsin although it rarely takes the stain, and it has lost its reactivity to Millon's reagent and mercury bromphenol blue. These reactions indicate that a chemical change is taking place in the sequence: blood mature morula cells, tunic mature morula cells, and *tunic vesicular cells.* This change appears to include a loss of protein material.

The dispersed vesicular cell in the tunic also displays changes in stain affinity which indicate that it is losing or depositing some material at the periphery of the tunic (Table I). Although the vesicles retain a P.A.S. reaction when fixed with cyanuric chloride and N-methyl morpholine, these vesicles lose their reactivity to alcian blue, colloidal iron, azure A at pH 4.0, never display a positive R.N.A. reaction when the cell is in the tunic, and lose their affinity for the protein stains (Table I). There also appears to be an increase in the M.B.E. point of the vesicles of this cell in the tunic.

Vacuolar blood cells in the tunics of *Clavelina lepadiformes* (Ries, 1937), *Pyura stolonifera* (Endean, 1955b), *Phallusia mammillata* (Endean, 1961), and *Ciona intestinalis* (Pérès, 1948b) contain a carbohydrate material as demonstrated by histochemical methods and are reported to be involved in tunic formation. Endean (1955b and 1961) states that the function of the vanadocyte and ferrocyte is tunicin secretion. In *H. aurantium,* although the mature morula cells deteriorate in the tunic as do the vanadocyte and ferrocyte, the mature morula cells consistently display negative reactions for either neutral or acidic polysaccharides. This suggests that the mature morula cells do not contribute to tunic carbohydrate. The staining reactions of the mature morula do indicate protein, and this protein stain affinity is lost by the time this cell degrades to a *tunic vesicular cell.* These reactions indicate that the mature morula cell may deposit protein in the tunic. The localization of the mature morula cells in body wall tunic just above the epidermis suggests that the function of these cells may be organizational in the sense that they contribute directly or indirectly to the fibers of tunic as they are secreted from the epidermis, organizing these fibers into definite layers or laminae (Smith, 1969).

III. Histochemistry of the tunic

There have been numerous reports of positive histochemical reaction of tunic for neutral carbohydrates or cellulose (St. Hilaire, 1931; Ries, 1937; Pérès, 1948a and 1948b; Endean 1955b and 1961; Deck *et al.*, 1966). It has been noted also that tunic is metachromatic (Pérès, 1948a and 1948b), and more recently it has

been reported that the tunic contains acidic polysaccharides or mucopolysaccharides (Endean, 1955a and 1961; Bierbauer and Vagas, 1962). The tunic matrix of *H. aurantium* gives a very weak (always less than cotton gauze or filter paper controls) to negative reaction with P.A.S. and stains as an acid mucopolysaccharide (Table III. The fibrous matrix of the tunic also has a positive histochemical reaction for protein. Chemical analyses of *H. aurantium* reveal that the tunic is approximately 50% protein and 50% carbohydrate by weight (Smith, 1969).

The cuticle and cuticular spines of *H. aurantium* show a positive reaction for some protein stains (Table III). Kennedy (1966) has reported that the cuticle of *Ascidia aspera* is scleroprotein. The negative reaction for sulfhydryl groups of the cuticular spines does not exclude the possibility of disulfides since the sections were not treated with a reducing agent prior to the histochemical reaction.

The reaction of the tunic matrix to acid mucopolysaccharide stains is more complex than is immediately apparent (Table III). Upon methylation and saponification there is a return of staining intensity with azure A at pH 1.5 to a level greater than the methylated section, but not to the level of control sections. Since carboxyl groups should be protonated at pH 1.5 and sulfate groups should be removed by methylation technique (Culling, 1963), the resurgence of staining

TABLE III

The histochemical reactions of the three tunic components, matrix fibers, spines, and epidermis. The following convention was used to indicate intensity of staining: − negative, ± faint positive, + definitely positive, ++ strong positive, +++ intense positive. Alpha, beta, and gamma refer to orthochromatic color, violet, and red metachromasia, respectively.

Reaction	Matrix Fibers	Spines	Epidermis
P.A.S.	± to −	−	−
Methylene blue extinction (pH)	pH 1.63	pH 4.28	pH 1.63
Toluidine blue	+++	+	+
	gamma	alpha	beta
Azure A pH 1.5	+++	±	++
Methylated	± to −	−	−
Saponified	++ to +	−	+
Azure A pH 4.0	+++	+	++
Methylated	±	+	+ to ±
Saponified	++	+	+
Aldehyde fuchsin	+++	±	++
Methylated	++	−	−
Saponified	++	−	+
Alcian blue	+++	−	+
Methylated	++ to +	−	+
Saponified	+	−	+
Colloidal iron	+++	−	+
Methylated	++ to +	−	±
Saponified	++ to +	−	±
DDD Sulfhydryl	−	−	±
Biebrich's scarlet	+	−	± to −
Millon's	±	±	±
Mercuric bromphenol blue	± to −	+	−
Methyl green pyronin (RNA)	−	−	+

intensity can be explained on the basis of incomplete methylation, exceptionally strongly dissociated carboxyl groups, or the presence of an uncharacterized substrate. The methylation period was 24 hours, so it is difficult to accept incomplete methylation as an alternative.

It has been reported that in *Perophora viridis* the epidermis will take up tritiated glucose which eventually appears in the tunic matrix, but that the vanadocyte does not accumulate the label (Deck *et al*, 1966). These observations and the histochemically analogous staining reactions of epidermis and tunic matrix in *Halocynthia* (Table III) are consistent with the hypothesis that the epidermis is the major tunic secreting system. This is reinforced by the fact that the epidermis of *Halocynthia* stains for the presence of R.N.A., as would be expected of a metabolically active tissue, whereas the mature morula and immature morula cells never display the presence of R.N.A.

IV. The effect of chemical and enzymic treatment on tunic histochemistry

The effects of hyaluronidase, pronase, and trypsin/papain digestion on tunic structure are summarized in Table IV. The epidermis and blood cells are destroyed by the preparative procedures. Morphologically there does not appear to be any change in matrix structure as a result of either proteolytic or hyaluronidase digestion. In *Pyura stolonifera* hyaluronidase treatment causes a disarrangement of fibrillar structure (Endean, 1955b), but in *Halocynthia* the orderly arrangement of fibers into laminae is unaffected by hyalurinidase digestion.

The cuticular spines undergo a limited degradation of structure upon hyaluronidase treatment and a greater degradation when treated with proteolytic enzymes (Table IV).

Acid, and acid followed by base, treatment of the tunic of *Halocynthia* completely destroys the cuticular spines, but there is no change in the orderly laminar structure of the fibrous matrix.

It is understandable that the tunic would furnish a negative P.A.S. reaction

TABLE IV

The effects of various experimental treatments on the structure and Alcian Blue-P.A.S. reactivity of the tunic fibers and spines. The foltowing abbreviations are used: A.B. alcian blue, P.A.S. Periodic Acid Schiff reaction, Hy'ase hyaluronidase, N. C. no change in the structure, N.R. no reaction, C.D. completely degraded, and P.D. partially degraded.

	Tunic Fibers			Tunic Spines		
	A.B.	P.A.S.	Remarks	A.B.	P.A.S.	Remarks
Treatment						
Control	+++	± to −	N.C.	—	—	N.C.
Tunicin prep.	±	−	N.C.	N.R.	N.R.	C.D.
6 *N* HCl 8 hours	+	−	N.C.	N.R.	N.R.	C.D.
Hyaluronidase	++	−	N.C.	−	−	P.D.
Pronase/Hy'ase	++	−	N.C.	−	−	P.D.
Trypsin/papain	++	−	N.C.	−	−	P.D.

if the carbohydrate component of tunic were masked by protein. Acid, acid and base, or proteolytic enzyme treatment should remove protein and reveal available P.A.S. reactive sites on the carbohydrate. However, subsequent to such treatment the tunic retains its overall negative reaction to P.A.S. (always less than cotton gauze or filter paper controls). Such chemical or enzymic treatment of tunic does decrease its affinity for alcian blue (Table IV) with the greatest decrease caused by acid and base treatment. If the carbohydrate component of tunic is a cellulose-like polymer, a possible explanation for the negative P.A.S. reaction after such chemical or enzymic digestion is extensive micelle formation due to hydrogen bonding between adjacent linear carbohydrate polymers (Cowling, 1963; Jayme and Lang, 1963) which denies access to the histochemical reagent molecules. This does not seem likely after strong acid and base treatment or if we consider that the P.A.S. reaction depends on a prior oxidation of the substrate with periodic acid. If the substrate molecule is not cellulose, then the failure of the P.A.S. technique can be explained on the basis of a lack of available vicinal diglycols which could be due to extensive cross-linking, deoxy sugars, or resistant glycosidic linkages through sugar carbons not involved in polymeric linkage.

These histochemical analyses of blood cells and tunic, coupled with quantitative evidence of discrete aggregation areas for the mature morula and dispersed vesicular cells in the tunic (Smith, 1969), allow certain hypotheses concerning the blood cells, tunic, and the function of the blood cells in the tunic.

This study does not support the hypothesis that the iron bearing cell contributes carbohydrate to the tunic, although it may contribute protein. The only tissue in *Halocynthia* which displays staining reactions that might indicate carbohydrate secretion is the epidermis. Further, the staining reactions of epidermis are analogous to those of the tunic matrix fibers which again would indicate that the epidermis is the tunic secreting tissue. However, the function of the mature morula cell must be closely allied to tunic formation since it displays aggregation and degeneration in the tunic just above the epidermis (Smith, 1969). The mature morula cell could be serving one of several functions in this position: it may be contributing protein to the tunic fibers as extruded from the epidermis, it may be instrumental in the formation of laminae or fibrous layers in the tunic (Smith, 1969), or it may be performing the latter function through the former. The histochemical reactions of the tunic matrix and cuticle and the resistance of tunic matrix to P.A.S., even after chemical or enzymatic treatment, indicate that the tunic is a complex chemical entity. If cellulose does exist in the tunic of *Halocynthia* it is, at least, heavily substituted. Preliminary investigations reveal that the tunic of *Halocynthia* contains significant amounts of hexosamine, is 50% protein, that serine and glucosamine are involved in the protein-polysaccharide linkage, and that the carbohydrate component of tunic has only a limited susceptibility to cellulase (Smith, in preparation). Consequently, the question of the composition of ascidian tunic, and more particularly the protein-polysaccharide composition, is still open.

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Summary

1. The similar staining properties of the vacuolar blood cells of *Halocynthia aurantium* indicate the relationship of the cell types constituting this group.

2. The mature morula cells and dispersed vesicular cells of *Halocynthia* do not stain for the presence of polysaccharides, but do stain for protein.

3. Changes in the staining properties of mature morula and dispersed vesicular cells between blood and tunic indicate that these cells contribute protein to tunic structures.

4. The tunic matrix and epidermis show stain affinities characteristic of acid mucopolysaccharide material.

5. The tunic matrix fibers display a consistent negative reaction to the P.A.S. technique. Neither enzymatic nor chemical treatment of tunic increases the reactivity of the fiber to P.A.S. reagent.

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